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13. ABSTRACT (Maximum 200 words) We have conceived of an approach to prepare by combinatorial methods, libraries of novel ligands for the estrogen receptor, that might be useful in the treatment or prevention of breast cancer, by the creation of simple amide or five-membered ring heterocyclic core structures that display peripheral substituents (phenols, aliphatic groups, etc.) commonly found in non-steroidal estrogens. We have made good progress on the preparation of novel estrogen of the diphenyl carboxamide class, the diphenylsulfonamide class, the phenyl benzylcarboxamide and sulfonamide classes, and the pyrazole, oxazole, thiazole, and imidazole classes. Members of same class have high affinity for the estrogen receptor. Future efforts will be directed at perfecting the solid phase synthesis of the pyrazole class, which appear to be the most promising, and expanding the size of the libraries that can be prepared.				
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21 Aug 1998

PI - Signature

Date

John A. Katzenellenbogen

TABLE OF CONTENTS

<u>Section</u>	<u>Page</u>
Table of Contents	4
Introduction	5
Body	6
Conclusions	14
References	14
Appendices	17

ANNUAL REPORT: September 1, 1997 – August 31, 1998**PRINCIPAL INVESTIGATOR: John A. Katzenellenbogen****TITLE: Combinatorial Synthesis For The Expedited Discovery Of Novel Selective Antiestrogens For Breast Cancer Prevention And Therapy****ORGANIZATION: University Of Illinois****INTRODUCTION AND GOALS OF THE PROJECT**

Antiestrogens such as tamoxifen are widely used in the treatment of hormone responsive breast cancer and recently shown to be effective in breast cancer prevention¹⁻⁵. Tamoxifen, however, as well as the pure ICI antiestrogens, are not ideal agents, because they cause vaginal atrophy and menopausal hot flashes, induce osteoporosis (pure antiestrogens), and may cause endometrial and liver cancer (tamoxifen)⁶⁻¹⁴. Thus, there is a need for the development of selective antiestrogens with an improved endocrine profile for use in the treatment and prevention of breast cancer¹⁵⁻¹⁶.

Recent advances in our understanding of the molecular pharmacology of estrogens and the development of new selective antiestrogens for menopausal bone maintenance, suggest that new selective antiestrogens of this type can be discovered¹⁶⁻²⁵. Up to now, however, this search has not been approached in a systematic fashion²⁶. Furthermore, the structures of antiestrogens that have been studied to date are quite complex and their synthesis sufficiently challenging, so as not to be amenable to synthesis by solid-phase combinatorial means. Combinatorial synthesis is the fastest growing new technology in pharmaceutical chemistry, and is proving to be a highly expeditious and promising approach to new drug discovery²⁷⁻³⁵.

In preparing for this project, we analyzed the structures of many selective antiestrogens and found that they possess three common peripheral groups (a phenol, a second aromatic group, and a basic side chain) attached to various core structures. Because the core structure appears to function as a scaffold simply to hold these other appendages together, we then designed six novel core structures that will perform the same scaffold function for the peripheral groups, yet are sufficiently simple that they can be readily prepared by solid-phase combinatorial synthesis.

In this project, we proposed to prepare six novel classes of ligands for the estrogen receptor, based on four functional group so far unexplored in the antiestrogen literature: a

carboxamide, a sulfonamide, a pyrazole, and an oxazole (and related thiazole and imidazole). Solution-phase syntheses were first to be developed, and then adapted to solid-phase synthesis using an acid-labile linker attached to the common phenol function. Libraries containing 180-1200 members of these six classes were then to be prepared, and all of these compounds then to be assayed for their binding affinity for the estrogen receptor. The estrogen agonist and antagonist activity of those members with high affinity was then later to be determined in cell transfection and proliferation assays, and those with the most appropriate endocrine activity tested in a uterotrophic assay.

The combination of a novel structural insight leading to the design of new core structures for estrogen receptor ligands that can be readily prepared by combinatorial synthesis, together with a set of simple, but effective assays to establish their hormonal activity, should assist in the discovery of novel selective antiestrogens for the treatment and prevention of breast cancer.

BODY

Experimental Approach

A General Structural Description of Selective Estrogen Receptor Ligands Suggests Alternate Core Structures that Can be Prepared by Solid Phase Combinatorial Chemistry Methods

As a class, selective antiestrogens can be envisioned as having four structural components (Schemes 1 and 2): *a core structure (A)* onto which are appended three other structural elements, *a phenol (B)*, *a second aromatic group (C)* and *a basic side chain (D)*. The achievement of an appropriate balance of estrogenic vs antiestrogenic activities in each of these series of selective antiestrogens appears to involve a delicate interaction between these component parts of their structure. Curiously, whereas components **B**, **C**, and **D** are rather similar in almost all of the selective antiestrogens (cf. Scheme 1, selective antiestrogens), the central core structure **A**, which links together the other three components like a scaffold, is quite variable. This suggests that other core structures could replace this central component in selective antiestrogens.

In the original proposal we proposed to explore new antiestrogens specifically designed to have novel core structures that are readily amenable to solid phase combinatorial synthesis and which may prove to be more tissue selective and efficacious for breast cancer. As explained in Scheme 3, we identified three simple structure motifs that are found in ER ligands, around which we designed six new classes of potential ligands for the ER that are based on four new core structures (Scheme 3, right column). We anticipated that these four core structures—a carboxamide, a sulfonamide, a pyrazole, and an oxazole (and a related thiazole and imidazole)—

would provide a suitable molecular scaffold for the other three components typically found in selective antiestrogens—the phenol, the second aromatic unit, and the basic side chain—so that these new structural classes would also have selective antiestrogenic activity. The unique feature of these new core structures is that, unlike antiestrogens prepared up to now, they can be prepared by combinatorial chemistry means.

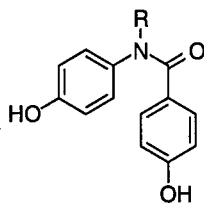
The first of the motifs, *motif A is an anti-bibenzyl system*, a structural subunit that is found in the potent estrogen hexestrol, as well as in the antiestrogen hydroxytamoxifen (Scheme 3). The structural analogs of this motif that we planned to explore, the diphenylcarboxamide and diphenylsulfonamide (Classes I and II), are well suited to three-component combinatorial solid phase synthesis methods (see below and Scheme 6). *Motif B is the homolog of the bibenzyl motif*, a substructure that is exemplified in the potent estrogen benzestrol and the selective estrogen raloxifene, but is otherwise largely undeveloped. We planned to explore several structural analogs of the homobibenzyl motif that are better suited for solid phase three-component combinatorial synthesis, benzylic homologs of the carboxamides and sulfonamides (Classes III and IV) and pyrazoles (Class V) (see below and Schemes 6 and 7). Finally, *Motif C, a syn-bibenzyl system* found in tamoxifen and centchroman, is the motivation for the three-component combinatorial synthesis of a series of heterocyclic analogs, oxazoles, thiazoles, and imidazoles (Class VI; see below and Scheme 8).

As we discuss below, we have made good progress evaluating the synthetic feasibility and ER binding affinity of members of all of these classes, as well as some others.

Results and Discussion

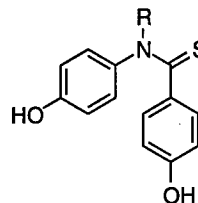
Synthesis of Class I: Diphenyl Carboxamides

We have followed the approach outlined in Scheme 6 in the original proposal to prepare a number of members of this class. Their structures are shown below, together with their binding affinity to ER. All of these compounds have been purified to analytical purity and fully characterized by spectroscopic means.



R	RBA
Et	0.062 %
Bu	0.042 %
<i>i</i> -Bu	0.02 %
<i>i</i> -Pr	0.009 %
Pentyl	0.007 %
Benzyl	0.089 %
CH(CH ₃)(CF ₃)	0.49 %
CH(C ₆ H ₅)(CF ₃)	10 %
CH(C ₆ H ₅)(CH ₃)	0.45 %

Estradiol = 100%



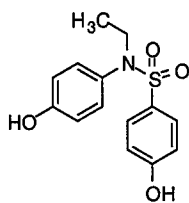
R	RBA
Et	0.62 %
Bu	1.3 %
<i>i</i> -Bu	1.5 %
<i>i</i> -Pr	0.83 %
Pentyl	1.7 %
Benzyl	0.15 %
CH(CH ₃)(CF ₃)	7.5 %
CH(CH ₃)(<i>i</i> -Pr)	4.6 %
CH(C ₆ H ₅)(CF ₃)	14 %
CH(C ₆ H ₅)(CH ₃)	2.5 %

It is interesting that the ER binding affinity of the members of this class cover a wide range, in a manner that is quite sensitive to relatively small changes in their structure. There are certain members that have quite high ER affinities.

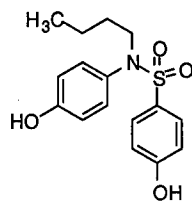
One new finding that we made was that the thioamides have higher ER binding affinities than the carboxamides. In addition, fluorine substituents on the N-alkyl group greatly improve binding affinity. This is thought to be a combination of a lipophilic effect and an electronic effect on the syn-anti ratio of the amide geometric isomers, an issue that is being studied further.

Synthesis of Class II: Diphenyl Sulfonamides

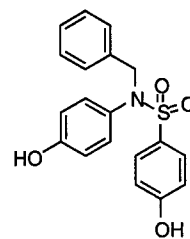
We have followed the approach outlined in Scheme 6 in the original proposal to prepare a number of members of this class. Their structures are shown below, together with their binding affinity to ER. All of these compounds have been purified to analytical purity and fully characterized by spectroscopic means. As a class, however, these compounds generally have quite low ER binding affinity, and because of this they are not being studied further.



RBA = 0.23 %



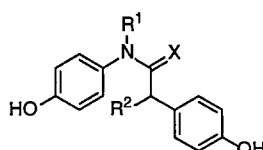
RBA = 0.13 %



RBA = 0.053 %

Synthesis of Class III: Phenyl Benzylcarboxamides and Sulfonamides

We have followed the approach outlined in Scheme 6 in the original proposal to prepare a number of members of this class. Their structures are shown below, together with their binding affinity to ER. All of these compounds have been purified to analytical purity and fully characterized by spectroscopic means.



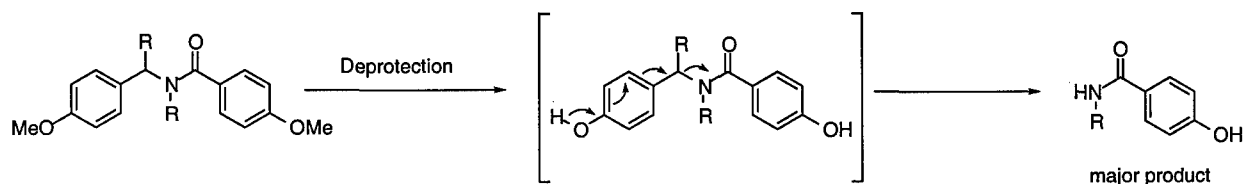
X	R ¹	R ²	RBA
O	Et	H	0.006 %
O	Et	Et	0.004 %
O	CH(CH ₃)(CF ₃)	CH ₃	0.01 % (1/1)
S	Et	H	0.020 %
S	Et	Et	0.089%

(Estradiol = 100%)

As indicated from the ER binding affinities this class appears to be unpromising at this time; however, we are continuing to analyze additional members containing the analogous substituents found in the higher binding analogs from Class I.

Synthesis of Class IV: Benzyl Phenylcarboxamides and Sulfonamides

We attempted to follow the approach outlined in Scheme 6 in the original proposal to prepare members of this class. However, we found that these compounds are unstable. An elimination reaction occurs whereby the amide unit is lost from the benzylic position of the *p*-hydroxybenzyl unit, with the presumed intermediacy of a quinone-methide, as illustrated below.

**Synthesis of Class V: Pyrazoles**

We have followed the approach outlined in Scheme 6 in the original proposal to prepare a number of members of this class. Their structures are shown below, together with their binding affinity to ER. All of these compounds have been purified to analytical purity and fully characterized by spectroscopic means.

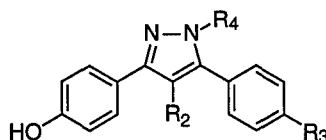


Table 1. RBA Data for Pyrazoles

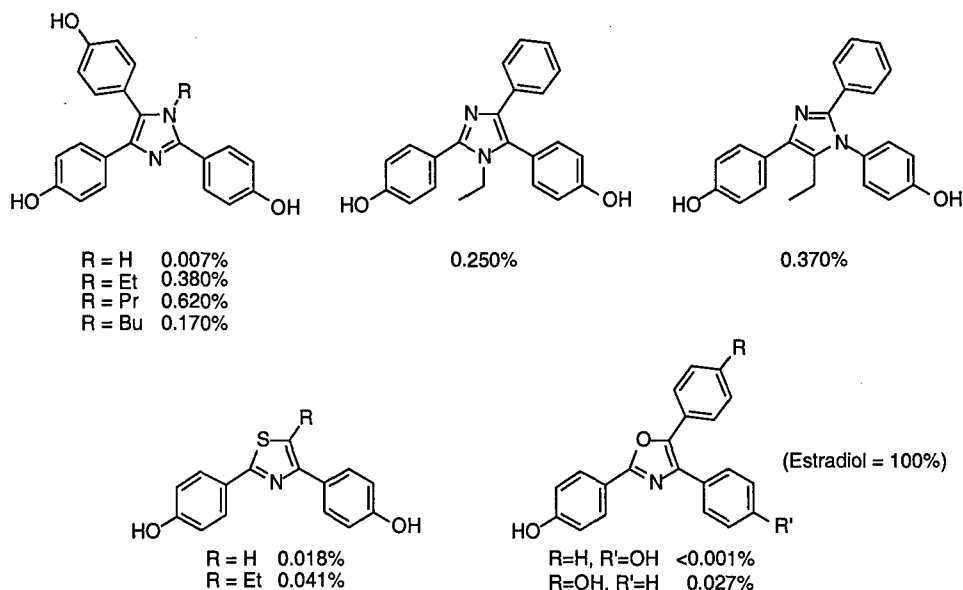
R ₂	R ₃	R ₄	% RBA
H	OH	H	0.009
H	OH	C ₆ H ₅	0.028
H	OH	C ₆ H ₅ CH ₂	<0.007
H	OH	p-HOC ₆ H ₄	0.059
C ₂ H ₅	OH	H	0.015
C ₂ H ₅	OH	C ₆ H ₅	14
C ₂ H ₅	OH	C ₆ H ₅ CH ₂	0.15 1.2 0.47
C ₂ H ₅	OH	p-HOC ₆ H ₄	2.4 11 22
CH ₃	OH	C ₆ H ₅	1.6
C ₂ H ₅	OH	CH ₂ CH ₂ OH	1.2
C ₂ H ₅	OH	(CH ₂) ₄ N((CH ₂) ₄)	0.013
C ₃ H ₇	OH	C ₆ H ₅	25
CH(CH ₃) ₂	OH	(CH ₂) ₄ N((CH ₂) ₄)	0.013

(Estradiol = 100%)

The binding properties of these compounds turned out to be very interesting as some of them have high affinity for the ER, and we have focused most of our attention on this class for the adaptation of syntheses to solid phase (see below).

Synthesis of Class VI: Oxazoles, Thiazoles and Imidazoles

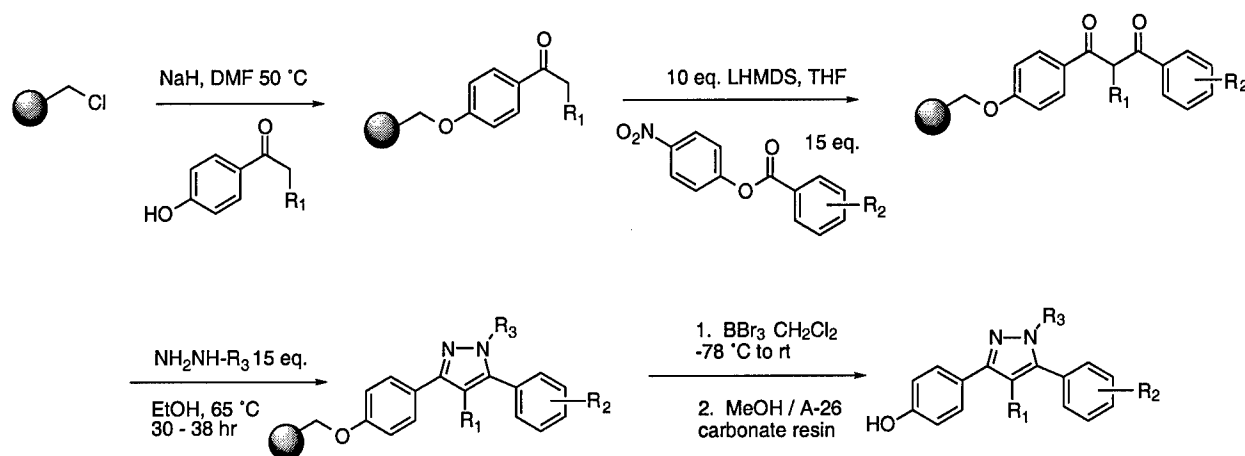
We have followed the approach outlined in Scheme 6 in the original proposal to prepare a number of members of this class. Their structures are shown below, together with their binding affinity to ER. All of these compounds have been purified to analytical purity and fully characterized by spectroscopic means.



We have been able to make a number of conclusions based on our results so far. High affinity binding of these heterocycles seems to require four ring substituents. This means that the oxazoles and thiazoles, which can bear a maximum of three substituents, will probably never afford high ER binding affinity. The imidazoles, which can accommodate four substituents, have the highest affinities of the group, but, compared to the pyrazoles (Class V), they are relatively poor binders. Thus, in addition to being able to bear four substituents, the nature of the core heterocycle is important. Whether the difference in affinity between the pyrazoles and the imidazoles is the result of differing magnitudes or directions of dipole moments or other factors is not yet understood and is being investigated further by the preparation of additional isomers and analogs.

Adaptation of the Synthesis of Class V Agents (Pyrazoles) to Solid Phase Methods

Because they are both the most unusual structurally and have the highest ER binding affinities, we have selected the pyrazoles (Class V) as the ER ligands whose synthesis we wish to explore by solid phase methods. We have modified the approach shown in the original proposal for solid phase methods to prepare members of Class V, the pyrazoles. Because rather vigorous conditions were required to deprotect the protecting group on the second phenol substituent, we chose to attach the first phenol to the resin directly through a benzyl ether linkage (Merrifield), rather than through the more acid-labile and expensive Wang resin. Thus, the overall scheme for resin attachment³⁶⁻³⁷, pyrazole synthesis, and cleavage/deprotection are shown in the Scheme below:



We have spent considerable time working out conditions to ensure that the loading of the resin proceeds in optimal yield, and we have used IR and NMR spectroscopy on polymer-bound material to monitor all of the transformations ³⁸⁻⁴⁰. We can follow the formation of the 1,3-diketone in the Claisen condensation using a ^1H NMR-MAS nanoprobe by observing the loss of the methylene proton signal α to the ketone and subsequent appearance of the methine signal at approx. 5 ppm (additional diagnostic chemical shifts are also prevalent). Formation of the pyrazole by reaction of hydrazine with dione can easily be followed by monitoring progressive disappearance of the carbonyl ($\text{C}=\text{O}$) band at 1670 cm^{-1} .

We have also worked to develop a consistent and efficient method to isolate the product pyrazoles free from inorganic contaminants and to characterize the level of residual synthetic precursors that are present in the sample. A standard, steep gradient elution of a reversed phase HPLC column proves to be a robust approach to characterizing product purity. Because the pyrazoles are uniformly fluorescent, we can use fluorescence detection to identify which of the eluted peaks is due to the pyrazole.

As we contemplate the synthesis of larger libraries, we have also evaluated various systems for the preparation of these pyrazoles on solid phase in a microtiter plate format. The Polyfiltronic plate system appears to work well, and withstands the solvent temperature combinations that are needed in the cyclization step of the pyrazole synthesis.

Progress in Relation to the Statement of Work

The complete three year Statement of Work, presented in the original proposal of July 1996, is shown below:

=====

ORIGINAL STATEMENT OF WORK

Project Period: July 1, 1997– June 30, 2000 (3 years)

Year 1

- Synthesis of representative members of Class I-VI ligands by solution phase methods.
- Isolate, purify, and fully characterize these members.
- Measure estrogen receptor binding affinity of representative members of Class I-VI ligands
- Begin to adapt solution phase syntheses to solid phase.

Year 2

- Complete adaptation of solution phase synthesis to solid phase.
- Isolate and fully characterize representative members produced by solid phase synthesis.
Determine yield and characterize impurities.
- Compare estrogen receptor binding of representative members of Class I-VI ligands prepared by solution vs solid phase.
- Begin synthesis of full Class I-VI libraries.
- Begin cell proliferation and cell-based transfection assays.

Year 3

- Complete synthesis of full Class I-VI libraries.
- Complete estrogen receptor assay of full libraries at two concentrations.
- Reassay the members with detectable estrogen receptor binding affinity by quantitative titration assay.
- Assay the members with high estrogen receptor binding affinity in the cell proliferation and cell-based transfection assays.
- Assay uterotrophic activity of the most promising members in rats.

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It is evident from the results presented in the preceeding sections that we have fulfilled all of the components of the Statement of Work for Year 1. In addition, we are already progressing on elements of the Statement of Work for Year 2, in that we have isolated and characterized representative members produced by solid phase synthesis, and we have determined yields and characterized impurities. We are beginning to make progress on the other work elements for Year 2.

CONCLUSIONS

From the results that we have achieved so far, we have identified several new series of non-steroidal estrogens that are adaptable to a combinatorial synthesis approach on solid phase. Some of the members of these series also have high affinity for the estrogen receptor. Thus, the project is progressing well along the lines that were originally envisioned in the initial proposal.

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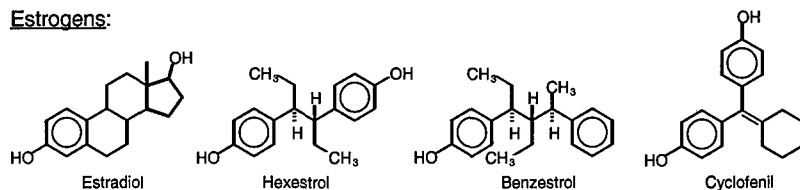
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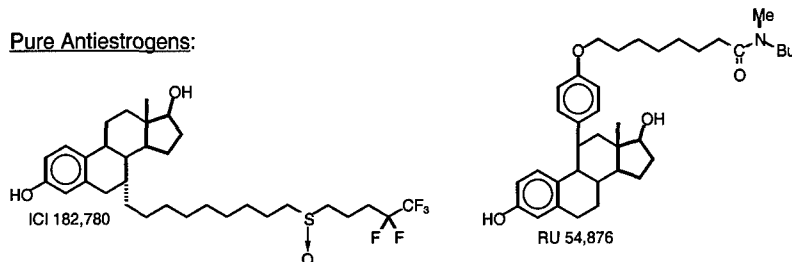
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Scheme 1. Estrogens, Pure Antiestrogens, and Selective Antiestrogens – From the Original Proposal

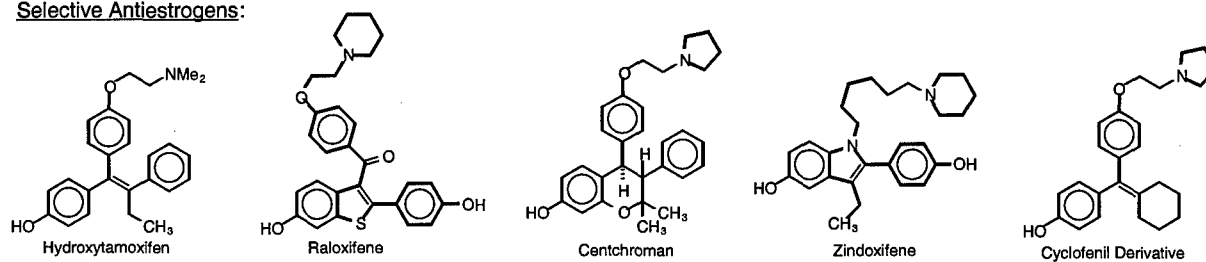
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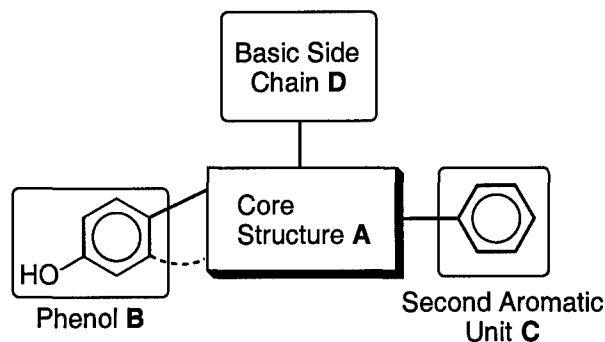
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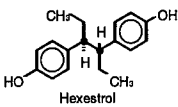
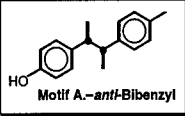
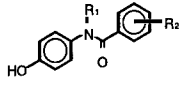
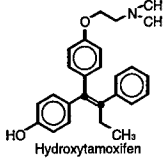

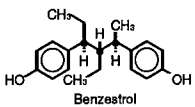
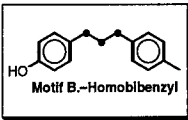
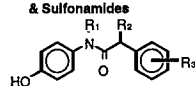
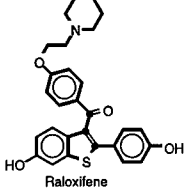
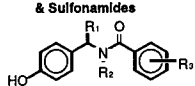
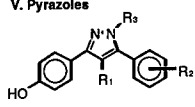
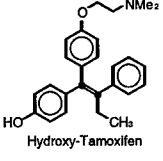
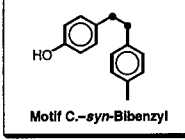
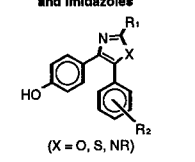
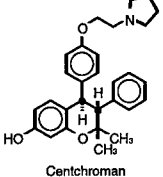
Selective Antiestrogens:



Scheme 2. Structural Components of Selective Antiestrogens – From the Original Proposal

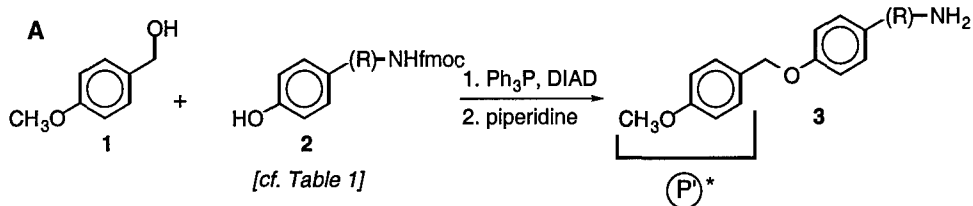


Scheme 3. Structural Motifs for Estrogen Receptor Ligands
and Their Combinatorial Analogs – From the Original Proposal

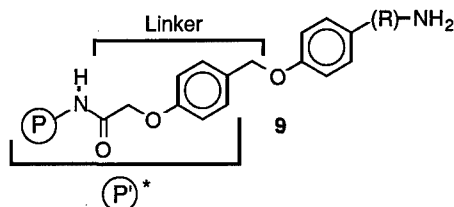
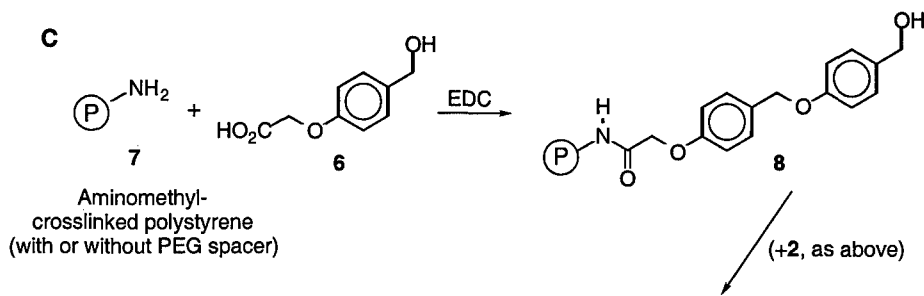
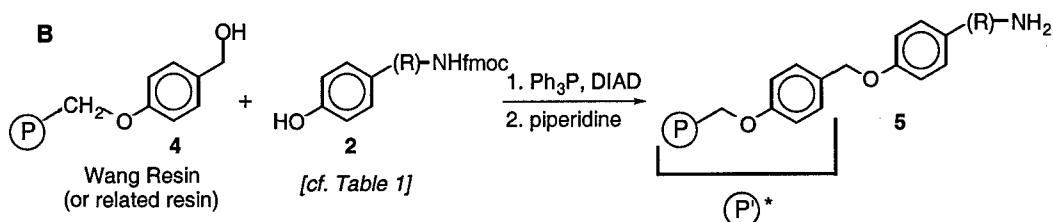
Estrogen Receptor Ligand	Structural Motif	Combinatorial Analog (Class)
 Hexestrol	 Motif A. <i>anti</i> -Bibenzyl	I. Diphenyl Carboxamides 
 Hydroxytamoxifen		II. Diphenyl Sulfonamides 
 Benzestrol	 Motif B. <i>Homobibenzyl</i>	III. Phenyl Benzylcarboxamides & Sulfonamides  (or SO ₂ in place of CO)
 Raloxifene		IV. Benzyl Phenylcarboxamides & Sulfonamides  (or SO ₂ in place of CO)
		V. Pyrazoles 
 Hydroxy-Tamoxifen	 Motif C. <i>syn</i> -Bibenzyl	VI. Oxazoles, Thiazoles, and Imidazoles  (X = O, S, NR)
 Centchroman		

**Scheme 5. Protection or Attachment of the Phenolic Amine Component (Element 1)
– From the Original Proposal**

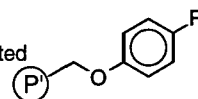
Element 1 Solution phase synthesis [protection]



Element 1 Solid phase synthesis [attachment]

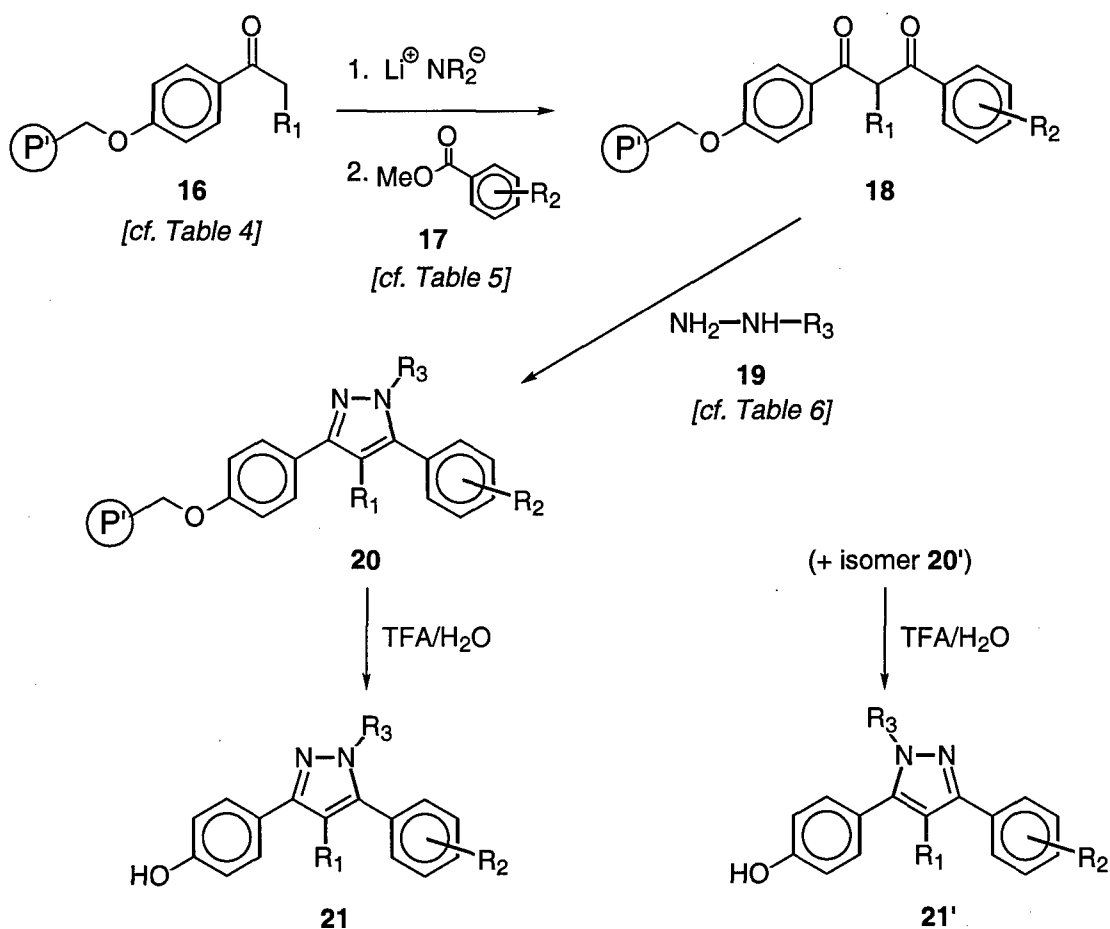
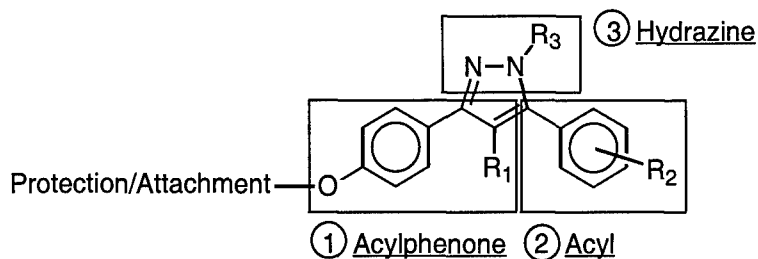


*In the following Schemes, the protecting group or polymer linker unit is designated



Scheme 7. Combinatorial Synthesis of Pyrazoles (Class V) – From the Original Proposal

Combinatorial Elements





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
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